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<b>(21) International Application Number:</b> PCT/US96/09726 <b>(22) International Filing Date:</b> 7 June 1996 (07.06.96)  <b>(30) Priority Data:</b> 08/479,328 7 June 1995 (07.06.95) US 08/580,980 3 January 1996 (03.01.96) US 08/644,116 10 May 1996 (10.05.96) US  <b>(71) Applicant:</b> LUDWIG INSTITUTE FOR CANCER RE- SEARCH [CH/US]; 1345 Avenue of the Americas, New York, NY 10105 (US).  <b>(72) Inventors:</b> PFREUNDSCHUH, Michael; Innere Medizin 1, D- 68187 Homburg (DE). RAMMENSEE, Hans-Georg; Im Neuenheimer Feld 242, D-69120 Heidelberg (DE).  <b>(74) Agent:</b> HANSON, Norman, D.; Felfe & Lynch, 805 Third Avenue, New York, NY 10022 (US).		<b>(81) Designated States:</b> AU, CA, CN, FI, JP, NO, NZ, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> METHOD FOR IDENTIFYING OR ISOLATING A MOLECULE AND MOLECULES IDENTIFIED THEREBY  <b>(57) Abstract</b>  The invention described methods for identifying a molecule of interest, as well as nucleic acid molecules which encode it, and binding partners for it. A cDNA library from a cell expressing the target is prepared, and expressed in host cells. Lysates of the host cells are screened with a sample, treated to remove interfering binding partners. The treatment involves contact of the sample to lysates of untransfected host cells, and host cells transfected or transformed with the same vector used to make the cDNA library. Also a part of the invention are antigens and cDNA identified using the methodology.		

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**METHOD FOR IDENTIFYING OR ISOLATING A MOLECULE  
AND MOLECULES IDENTIFIED THEREBY**

**RELATED APPLICATIONS**

This application is a continuation-in-part of Serial No. 08/580,980, filed on January 3, 1996, which is a continuation-in-part of application Serial No. 08/479,328, filed on June 7, 1995, both of which are pending, and are incorporated by reference.

**FIELD OF THE INVENTION**

This invention relates to methodologies for identifying molecules of interest. In particularly preferred embodiments, the invention relates to the identification of molecules associated with pathological conditions such as cancer, (melanoma or renal cancer, e.g.), Hodgkin's Disease, autoimmune diseases and so forth. Also a part of the invention are the isolated molecules found as a result of the inventive method, such as presented peptides. These molecules include, inter alia, protein-containing molecules, isolated nucleic acid molecules encoding these, and antibodies which specifically bind to the protein-containing molecules. For convenience, the method described herein will be referred to as "serological fishing".

**BACKGROUND AND PRIOR ART**

It is fairly well established that many pathological conditions, such as infections, cancer, autoimmune disorders, etc., are characterized by the inappropriate expression of certain molecules. These molecules thus serve as "markers" for a particular pathological or abnormal condition. Apart from their use as diagnostic "targets", i.e., materials to be identified to diagnose these abnormal conditions, the molecules serve as reagents which can be used to generate diagnostic and/or therapeutic agents. A by no means limiting example of this is the use of cancer markers to produce antibodies specific to a particular marker. Yet another non-limiting example is the use of a peptide which complexes with

an MHC molecule, to generate cytolytic T cells against abnormal cells.

Preparation of such materials, of course, presupposes a source of the reagents used to generate these. Purification from cells is one laborious, far from certain method of doing so. Another preferred method is the isolation of nucleic acid molecules which encode a particular marker, followed by the use of the isolated encoding molecule to express the desired molecule.

To date, two strategies have been employed for the detection of such antigens in, e.g., human tumors. These will be referred to as the genetic approach and the biochemical approach. The genetic approach is exemplified by, e.g., DePlaen et al., Proc. Natl. Acad. Sci. USA 85: 2275 (1988), incorporated by reference. In this approach, several hundred pools of plasmids of a cDNA library obtained from a tumor are transfected into recipient cells, such as COS cells, or into antigen-negative variants of tumor cell lines which are tested for the expression of the specific antigen. The biochemical approach, exemplified by, e.g., Falk et al., Nature 351: 290 (1991), and Kawakami et al., Nature 369: 69 (1994) both of which are incorporated by reference, is based on acidic elution of peptides which are bound to MHC-I molecules of tumor cells, followed by reversed-phase high performance liquid chromatography (R-HPLC). Antigenic peptides are identified after they bind to empty MHC-I molecules of mutant cell lines which are defective in antigen processing, and induction of specific reactions in cytolytic T-lymphocytes. These reactions include CTL proliferation, TNF release, and lysis of target cells, measurable in an MTT assay, or a  $^{51}\text{Cr}$  release assay.

These two approaches to the molecular definition of antigens have the following disadvantages: first, they are enormously cumbersome, time-consuming and expensive; second, they depend on the establishment of cytolytic T cell lines (CTLs) with predefined specificity; third, their relevance in vivo for the course of the pathology or disease in question

has not been proven, as the respective CTLs can be obtained not only from patients with the respective disease, but also from healthy individuals, depending on their T cell repertoire.

5       The problems inherent to the two known approaches for the identification and molecular definition of antigens is best demonstrated by the fact that both methods have, so far, succeeded in defining only very few new antigens in human tumors. See, e.g., van der Bruggen et al., Science 254: 1643-1647 (1991); Brichard et al., J. Exp. Med. 178: 489-495 (1993); Coulie, et al., J. Exp. Med. 180: 35-42 (1994), Kawakami et al., Proc. Natl. Acad. Sci. USA 91: 3515-3519 (1994).

15       It would be desirable to have available a method which can be used not only for detection of tumor-associated antigens, but to determine molecules associated with any abnormal or pathological condition. Such a method would also facilitate the identification of such molecules, thereby enabling their use on the generation of, e.g., antibodies, cytolytic T cells, and so forth.

20       It is therefore the purpose of the present invention to develop methods and reagents for the simple detection and molecular characterization of antigens in human tissues, especially in tumor cells, which are useful in the molecular diagnosis of diseases and/or for immunotherapy and gene therapy of infectious, autoimmune and malignant diseases. The invention is delineated in the disclosure which follows.

#### BRIEF DESCRIPTION OF THE FIGURES

30       Figure 1 shows the principles of the approach of the invention.

Figure 2 shows a nitrocellulose membrane with a positive clone derived from the cDNA of a renal cell clear carcinoma that reacts with a 1:100 dilution of the patient's serum.

35       Figure 3 shows, in bar graph form, the Northern Blot analysis of clone HOM-RCC-313 in renal cell carcinoma, normal kidney and other human tissues.

Figure 4 shows the translated region of the gen coding for HOM-RCC-313.

#### DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

5 The following disclosure describes a methodology referred to as serological fishing. In it, a cell sample is taken from a subject afflicted with a pathological condition. The cells preferably are exemplary of the pathology. For example, if the subject has melanoma, the cells are melanoma cells. If the subject is suffering from a neural disorder, e.g., then  
10 the cells are preferably a sample of the afflicted cells. This approach is warranted because the afflicted cells are most probably the best source of protein-containing molecules of interest, i.e., such molecules which are specifically associated with the pathological condition of interest.

15 Note that cells representative of pathological conditions are not the only cells which may be used in the inventive method. It is very important, e.g., to ascertain those cellular "markers" associated with differentiation and maturation of cells, for example. The example of  
20 hematopoietic stem cells comes to mind. Similarly, the invention contemplates the isolation of, e.g., receptor molecules for specific ligands. In effect, one can assay for the presence of any molecule of interest using this methodology.

25 The cells chosen are then used to prepare a library of complementary DNA (i.e., "cDNA"). This methodology is well known to the skilled artisan, and need not be reiterated here. It is, of course, based upon the established fact that if proteins are expressed by the cells, then messenger RNA (mRNA)  
30 must be present. These mRNA molecules are not long lived, and are unstable, so they are not practical to work with. The stability brought to the molecules when cDNA is used is very helpful to the method.

35 Once the cDNA is made, it is used to construct a vector library. In short, carrier vectors are treated, such as by cutting and splicing, to receive molecules f cDNA. The

choice of vector may vary, as the skilled artisan is well familiar with many such examples.

Especially preferred are virus based vectors. In the case of eukaryotic cells, retrovirus or adenovirus based vectors are preferred. Such vectors contain all or a part of a viral genome, such as long term repeats ("LTRs"), promoters (e.g., CMV promoter, SV40 promoter, RSV promoter), enhancers, and so forth. When the host cell is a prokaryote, then bacterial viruses, i.e., phages, are preferred. Exemplary of such vectors are vectors based upon, e.g., lambda phage. In any case, the vector may comprise elements of more than one virus.

The resulting vectors are transfected or transformed into a host cell, which may be eukaryotic or prokaryotic.

Any cell normally used for transfection or transformation may be used in the protocol. Preferred materials include strains of *E. coli*, CHO cells such as CHO-1, COS cells such as COS-7, and so forth. Similarly, yeast cells, e.g., strains of *Saccharomyces*, strains of *Pseudomonas*, such as *Pseudomonas aeruginosa*, *Bacillus* bacteria, well known insect host cell *Spodoptera frugiperda*, and so forth, may all be used.

Once the recipient cells receive the vectors, they are cultivated so as to express foreign, protein containing molecules. "Protein-containing" is used herein because, while prokaryotes express only proteins, eukaryotic cells are well known for their ability to post-translationally modify proteins, so as to produce glycoproteins, lipoproteins, e.g. It must also be borne in mind that "protein containing" as used herein, also encompasses peptides, such as the peptides presented by MHC molecules.

The processes now described below take place independently of the process described above, and no chronological relationship between the two facets of the invention is intended.

In pathological conditions such as cancer and, e.g., autoimmune diseases, there is some immune reaction to molecules associated with the pathology. This reaction can

include an antibody response, B cell proliferation, proliferation of specific T cell subpopulations, increases in cytokine production, and so forth. The molecules and cells associated with the response may be found in body fluids of a subject, such as his or her serum. The immune responders will react with the molecule of interest whether it is produced recombinantly or autologously. The problem is to find them. As the examples show, this is done in a unique way. First, the body fluid, or other sample of interest, is reacted with a sample of the same host cells used for transfection or transformation. In this first step, the host cells are not transfected or transformed. The effect of this is to strip any immunogenic binding partners specific for the host cell rather than the targeted molecule. This step is necessary because, as was pointed out, supra, the host cell may be one against which the subject has developed an immune response at some point. This first stripping removes these immune components.

A second stripping step is then carried out. In this step, the previously stripped sample is now reacted with a sample of the same host cell as was described supra, this time having been transfected or transformed with the carrier vector lacking cDNA from the subject. The reason for this second stripping step is an observation made by one of the present inventors and not reported in the literature previously. The materials used as vectors, such as phages, viruses, etc., are useful because they naturally infect cells. Thus, E. coli, which inhabit the lower intestine of humans, are infected with lambda phage. It had not been considered, previously, that the immune response to E. coli includes a response to these infectious agents. Thus, applicants have surprisingly, achieved an ability to remove interfering immune components to an unprecedented degree by carrying out the two stripping steps. As noted, the first is against untransfected or untransformed host cells. The second is against host cells transfected or transformed with a vector which does not carry



cDNA, wherein the vector is immunologically equivalent to the vector used to carry cDNA, as described supra.

It is especially preferred to carry out each of these stripping steps using a plurality of similar, but different procedures. The experiments which follow, for example, show absorption on a solid phase column, and then absorption on nitrocellulose paper. Applicants do not wish to be bound by any theory as to why the use of two similar but different protocols produces the results described herein. It is to be borne in mind, hereafter, that whenever "contacting a sample" is used herein, it is not to be limited to one contact step only, but may refer to more than one, preferably different, contact protocols designed to remove interfering binding partners from a sample under scrutiny.

It should be understood that these stripping steps may be done completely independently of the steps used to prepare the cDNA library. For example, if the test for an antigen is to be done at day "0", the stripping of sample may be done the day before, a week before, and so forth. One can also "bank" stripped sample from a donor or subject for future use.

The sample used is preferably serum, but need not be. Any sample which contains immunogenic binding partners may be so used.

In the next step of the method, lysed, transfected cells carrying the cDNA and expressing heterologous protein are contacted with the twice stripped sample. This sample should only contain immune components specific for the heterologous protein, and should bind thereto. This binding is facilitated if the cell lysates have been immobilized via contact to, e.g., activated filter paper, a solid phase column, etc., but this solid phase binding is not necessary, as the art will surely recognize that many, varied forms of assays are available for identifying a molecule of interest.

Once the immune component binds to the target molecule, a further step is desirably, but not necessarily, carried out. This additional step involves the use of some binding partner for the first immune component, such as anti-IgG, carrying an

identifiable label. The label may be a dye, an enzyme, a gold particle, a radiolabel, or any of the standard lab ls used in immunoassays.

5 Once identification is carried out, the immune components are removed, leaving the target molecule. The target molecule is then studied, using any of the standard methodologies in the art.

10 The artisan will note that the methodology also results in isolation of immune components which bind to the molecule of interest. Thus, in another aspect of the invention one can isolate antibodies, e.g., which are specific binding partners for the molecule of interest.

15 Yet another immune component which may be identified and isolated following the invention is a cytolytic T cell ("CTL" hereafter), specific for complexes of peptides derived from the identified molecule and MHC molecules to which these peptides bind, forming a complex. It is fairly well accepted that a CTL response involves the identification of complexes of MHC molecules and peptides, generally about 8-12 amino  
20 acids in length, but most preferably 9 or 10 amino acids in length, by T cell receptors ("TCRs") on the surface of circulating T cells. The TCRs react by binding to these complexes, "setting in motion," as it were, a series of reactions including the proliferation of CTLs specific for these complexes. One can produce and/or isolate such CTLs  
25 using the method of the invention, plus further steps.

As is pointed out in the examples which follow as well as the disclosure in general, one can easily identify cDNA encoding an antigen of interest. Once the cDNA is identified,  
30 one uses it to transfect host cells which either already present desired MHC molecules on their surface, or which have been transfected with DNA encoding these MHC molecules. The cDNA for the molecule of interest is expressed, and the molecule is processed to antigenic peptides which are  
35 presented by MHC molecules, such as HLA molecules. CTLs directed against the complexes are obtained from lymphocytes, such as autologous lymphocytes. From r sponder cell

populations, long-term CTL clones are then obtained by the well known technique of limiting dilution. Once a positive CTL response is observed, the specific peptides presented to the CTLs are identified using established methods for example, screening the specific of previously identified CTL clones. Alternatively, the more recently described method of studying the sequence of the molecule of interest to identify potential MHC-binding motifs then analyzing these peptides, first for binding to the relevant MHC molecule and then, if positive for MHC-binding, for their ability to generate CTLs recognizing the peptide MHC complex may be used. Of course the peptides can also be eluted from the cells and sequenced, using well known techniques.

It will also be noted by the skilled artisan that one can correlate the expression of the molecule of interest back to a particular host cell or cells which expressed it. In so doing, one can remove the cDNA which expressed the molecule of interest, sequence it, and so forth. This aspect of the method is another feature of the invention.

Specific embodiments of the invention will be seen in the examples which follow. Figure 1 depicts the method generally.

#### Example 1

For the establishment of a cDNA library from human tissue total RNA was obtained from 0.5 ug of a renal clear cell carcinoma and established according to the method of Chomzynski, J. *Analyt. Biochem.* 162: 156-159 (1987), incorporated by reference. The mRNA was extracted from total RNA with oligo-dT-cellulose. The synthesis of the first strand cDNA was accomplished by the method described by Gubler and Hoffmann, *Gene* 25: 263 (1983) using RNase H and DNA polymerase I. For adaptation of the cDNA Klenow enzyme, adaptors with EcoRI restriction enzyme sites were ligated to the cDNA ends using T4 DNA ligase (Ferretti L and Sgamerella V, *Nucl. Acids Res.* 9: 3695 (1981)). Following restriction enzymatic digestion with the enzyme XhoI, cDNA molecules of different length were separated using Sephacryl 400 and

transfected into  $\lambda$ ZAPII phage vectors (Short et al., Nucleic Acids Res. 16: 7583 (1988)). The recombinant phage DNA was packed into phages after ligation with packing extracts and used for the transfection of *E. coli* bacteria. The titration of the library resulted in  $1.8 \times 10^6$  recombinant primary clones. The total cDNA library was transfected in *E. coli* and amplified. The titer of the cDNA library after amplification was  $10^{11}$  plaque forming units per ml (pfu/ml). These transfected cells were used in experiments which follow.

#### Example 2

In accordance with the invention as described, *supra*, identification of immunogenic material was achieved by using human sera which have been completely depleted of antibodies directed against antigens derived from native and lytic  $\lambda$  phage-transfected *E. coli* bacteria. To this end, the serum was "stripped" via absorption, as now described.

*E. coli* bacteria of the strain XL1-Blue were cultured in 50 ml LB medium overnight. After achieving an optical density of  $OD_{600} = 1.0$ , the bacteria were pelleted by centrifugation, resuspended in 5 ml phosphate buffered saline (PBS), and sonicated by ultrasound to form a lysate. The bacterial lysate was bound onto a matrix of activated Sepharose, which was then put into a column and used for the absorption of the human serum. The serum was run over this column 10 times.

A culture of *E. coli* XL1-Blue bacteria in the exponential growth phase was pelleted by centrifugation, transfected in 0.01 M magnesium sulfate with  $10^6$   $\lambda$ ZAPII phages without a recombinant insert and incubated in 5 ml LB medium for four hours. The lysate of the transfected bacteria was used in the same manner as the untransfected bacteria, with the human serum described *supra* being passed through the column an additional ten times.

To complete the depletion of the serum, interfering antibodies from lytically transfected *E. coli* bacteria were cultured on agar plates (10 hours, 37°C) and their proteins were blotted onto nitrocellulose membranes after this

culturing step. Following this, the serum which had been preabsorbed according to the above steps was transferred to the blotted nitrocellulose membrane, and the absorption procedure was repeated five times. The serum, which was processed in accordance with the invention, was totally depleted of antibodies directed against antigens derived from *E. coli* and phages.

### Example 3

In these experiments, a renal cancer-specific antigen was identified via the following steps. Bacteria of the strain *E. coli* XL1-Blue were transfected with recombinant phages derived from the described cDNA library and plated at a density of  $4-5 \times 10^5$  plaque forming units (PFUs) per plate in LB-medium with isopropylthiogalactopyranoside ("IPTG"). After 12 hours of incubation at 37°C, nitrocellulose membranes were put on top of the cultures, and the culture plates were incubated for another four hours. This was followed by incubation of the nitrocellulose membrane for one hour in Tris-buffered saline (TBS) with 5% milk powder. After washing the nitrocellulose membranes three times in TBS, the stripped human serum secured following Example 2 was diluted 1:1000 in TBS/0.5% milk powder (w/v) and incubated overnight with gentle shaking. After the incubation with the nitrocellulose membrane the serum was removed and kept for additional testing. Following incubation with serum, the nitrocellulose membranes were washed three times in TBS, and incubated with polyclonal alkaline phosphatase-conjugated goat anti-human IgG for one hour. Following this, the nitrocellulose membranes were washed repeatedly with TBS/0.01% Tween 20 (v/v)). The reaction was developed using nitroblue tetrazolium chloride and bromochloro-indoyl-phosphate in TBS. The binding of human antibodies to the expressed protein became visible by a blue, ring-formed color deposit on the nitrocellulose membrane. The efficient preabsorption of the serum made it possible to develop the membrane at 37°C over several hours without compromising the quality of the test because of background

reactivity caused by antibodies against *E. coli* and phage antigens.

Positive clones were localized on the agar plates, transferred into transfection buffer, and used for a second round of transfection and subcloning. A total of  $1.8 \times 10^6$  recombinant clones were subjected to screening and five different positive-reacting clones were identified.

#### Example 4

Positive clones secured following Example 3, i.e., those which had bound antibodies derived from the processed human serum, were subcloned to monoclonality by repeated rounds of transfection and testing of reactivity with the processed human serum. P-bluescript phagemids with the respective cDNA inserts were cloned by *in vivo* excision (Hay and Short, Strategies 5: 16-19, 1992) from the  $\lambda$ ZAPII phage vectors and used for transfection of *E. coli* SOLR bacteria. Plasmids were isolated from the bacteria after alkaline lysis with NaOH in a modification of the method of Birnboim and Doly, J. Nucl. Acids Res. 7: 1513 (1979). The recombinant plasmid DNA was sequenced according to the classic method of Sanger (Proc. Natl. Acad. Sci. USA 74: 5463 (1977)) using M13-forward and M13-reverse oligonucleotides. The DNA sequence obtained and the resulting amino acid sequence were checked for in nucleic acid and protein data banks (Gene Bank, EMBL, Swiss Prot). The sequencing of the cDNA inserts was continued using internal oligonucleotides. Analysis showed no homology with any sequences deposited in the data banks. The full length cDNA clone referred to as SK313, which had been cloned with the RACE method (Frohman MA, Dush MK, Martin GR, Proc. Natl. Acad. Sci. USA 85: 8998 (1988)), had a carbonic anhydrase domain at the 5' end. The nucleic acid sequence of this molecule is presented in SEQ ID NO: 1. Figure 2 shows a nitrocellulose membrane with a positive clone from these experiments.

#### Example 5

As a follow up to these experiments, RNA was isolated from a spectrum of malignant and normal human tissues

according to the method of Chomzynski and Sacchi, *Analyt Biochem.* 162: 156 (1987). After denaturation, the total isolated RNA was separated on an agarose gel containing 1% formaldehyde by electrophoresis (Goldberg, *Proc. Natl. Acad. Sci. USA* 77: 5794 (1980)) and then blotted onto a nylon membrane according to a known method (Seed, *Nucl. Acids Res.* 10: 1799 (1982)). Radiolabeled cDNA inserts of the identified clones were used for hybridization. The hybridization was carried out according to a known method (Geoffrey and Berger, *Enzymol.* 152: 419 (1987)). The presence of the respective RNA was demonstrated using autoradiography and X-ray films. The analysis demonstrated that the mRNA of clone HOM-RCC-313 was overexpressed in 4 out of 19 renal cell carcinomas compared to normal kidneys. Very weak expression was found only in colonic mucosal tissue and in normal kidney. Expression in other tissues could not be demonstrated.

#### Example 6

To determine the incidence of antibodies against antigens which are identified in accordance with the invention, sera from healthy individuals and tumor patients were analyzed. To this end, the sera were processed as described, supra, and depleted of antibodies against antigens derived from *E. coli* and phages. For the detection of antigen-specific antibodies, phages derived from reactive clones were mixed with non-reactive phages derived from the same cDNA library at a ratio of 1:10 and tested as described supra, for reactivity with antibodies in the human test serum. The serum which had been used for the identification of the antigen was used as a positive clone. The non-reactive phages served as a negative control. A serum sample was positive for antigen reactive antibodies, if the expected percentage of the phage plaques showed a positive reaction. In the case of the renal cell carcinoma antigen represented by clone HOM-RCC-313, the analysis of a spectrum of human sera showed that only sera from renal cell carcinoma patients contained reactive antibodies. Sera from healthy controls and patients with other tumors did not contain such antibodies.

The cDNA for clone HOM-RCC-313 was excised from the plasmid DNA by digestion with the restriction enzyme EcoRI, and separated by agarose gel electrophoresis, followed by extraction from the gel. This was then used to create a vector which expresses a fusion protein with the bacterial protein anthranilate synthetase. A relevant fragment in the exact open reading frame was cloned into pATH plasmid vectors (Koerner, et al, Meth. Enzymol. 194: 477 (1991). Induction of protein expression was obtained after transformation of the plasmids into *E. coli* of strain BL21 as described (Spindler, et al, J. Virol. 49: 132 (1984)). Expressed, fusion proteins were separated by SDS gel electrophoresis, excised from the gel, eluted and freeze dried. Rabbits were immunized by subcutaneous injection with 100  $\mu$ g of the lyophilisate dissolved in Freund's adjuvant. Immunization was repeated three times at two-week intervals using incomplete Freund's adjuvant. The rabbit was bled and antiserum was obtained. The obtained antiserum was depleted from antibodies reactive with *E. coli* and phages in the manner described supra and tested for reactivity against the renal carcinoma antigen as described for the human serum. Reactivity was detected at dilutions of 1: > 100,000.

#### Example 7

The protocols set forth in the preceding examples were followed, using biopsied tissue taken from different subjects suffering from (i) malignant melanoma, (ii) astrocytoma, and (iii) Hodgkin's Disease. Table 1, which follows, summarizes the results, including those obtained with the renal cancer study, set out in detail in Examples 1-6, supra.



5 Table 1. Antibody reactivity of autologous sera with recombinant clones derived from human tumor cDNA. cDNA libraries were screened with autologous patient serum. Positive clones were subcloned to monoclonality. Inserts from each clone were amplified with plasmid primers and separated by agarose gel electrophoresis. Southern blots were performed by cross hybridization with the respective inserts.

10	tumor	clones tested	positive clones	different inserts
	malignant melanoma	$1.0 \times 10^6$	40	10
	renal cell carcinoma	$1.8 \times 10^6$	7	5
	astrocytoma	$1.2 \times 10^6$	49	5
15	Hodgkin's disease	$1.0 \times 10^6$	14	4

20 Analysis of the different inserts showed that the melanoma cells expressed the known tumor rejection antigen precursor MAGE-1 (see van der Bruggen et al., Science 254: 1643-7 (1991), incorporated by reference), as well as a new antigen. A portion of the cDNA sequence of this antigen is set forth in SEQ ID NO: 2.

25 When the astrocytoma study was completed, the observed insert appeared to correspond to the previously described Tegt gene (Old, Canc. Res. 41: 361-375 (1981), incorporated by reference).

30 When the Hodgkin's Disease study was completed, a previously unknown antigen was isolated, and cDNA encoding it was identified in the library, using standard methods. The antigen is a newly observed, lectin-like structure, a portion of the cDNA for which is set forth in SEQ ID NO: 3. Also observed were antibodies against restin, described by Bilbe, et al, EMBO J 11: 2103-13 (1992). This is an intermediate filament associated protein, expression of which has been shown to be restricted to Hodgkin and Reed-Sternberg cells, as well as cultured monocytes.

35

Example 8

A further study of occurrence of antibodies against the antigens described in Examples 1-7 was carried out. Table 2 summarizes these assays. In these studies, phages from positive clones were mixed with non-reactive phage (ratio:1:10), and then used to transfect bacteria (e. coli). Dilutions of patient sera (1:200), were used, in an enzyme linked immunosorbent assay (ELISA), as described supra. "HOM-MEL-40" refers to the new melanoma antigen (SEQ ID NO: 2), while "HOM-MEL-55" refers to MAGE-1 (van der Bruggen et al., supra). "HOM-RCC 3.1.3" is the renal cancer antigen of SEQ ID NO: 1. "HOM-GLO-30.2.1" refers to the previously identified astrocytoma associated antigen, "HOM-HD-21" refers to the new, lectin-like antigen of SEQ ID NO: 3, and "HOM-HD-397" is the previously identified restin antigen.

**Table 2.** Humeral immune responses against human tumor antigens. Phages from positive clones were mixed with nonreactive phages of the cDNA-library at a ratio of 1:10 and used to transfect bacteria. IgG antibodies to the clones were detected with an anzyme-linked assay using sera. n.t.

antigen	HOM-MEL-40+	HOM-MEL-55	HOM-ROC-3.1.3+	HOM-GLIO-30.2.1	HOM-HD-21+	HOM-HD-397
identify/homology	MAGE-1	CAH-like	tegt	lectin-like	restin	
melanoma patients	2/11	4/11	n.t.	n.t.	n.t.	n.t.
renal cancer patients	0/8	0/8	2/14	0/7	0/7	5/7
astrocytoma patients	0/10	0/10	0/11	2/13	0/11	7/11
Hodgkin's patients	0/10	0/10	0/17	0/17	10/18	14/17
healthy controls	0/12	0/12	0/15	0/20	0/17	12/17

The fact that antibodies against the tumor antigens, excepting only restin, were detected, albeit at varying rates, only in the sera of patients diseased with the same type of tumor suggests that tumor growth is essential for the development of a humoral response against tumor antigens.

The reason for the presence of restin in healthy controls is not clear. One may speculate that tolerance against respective antigens might be circumvented, because the antigen may have similar sequences to another antigen, the donor may have premalignant cells, or the antigen may be activated in normal cells under non-malignant conditions, such as viral infections, or other inflammatory processes.

#### Example 9

In order to determine the expression pattern of the newly identified antigens described herein, Northern blot analysis was carried out, using a variety of human tissues.

RNA was extracted from tissue samples (tumor and normal) using the well known guanidium isothiocyanate/phenol/chloroform method of Chomzynski, et al., supra. The RNA integrity was checked via electrophoresis in formalin/MOPS gels. Then, gels containing 40 ug of RNA per lane were blotted onto nylon membranes. These Northern blots were then probed with the cDNA of SEQ ID NO: 1, 2 or 3. Hybridization was with <sup>32</sup>P labelled probes at 42°C, with formamide. The filters were washed at 65°C, at 1xSSC, 0.2% SDS, and exposed for 16 hours. These are "stringent conditions" as defined hereafter. After exposure, filters were stripped and rehybridized with GAPDH.

Table 3 summarizes these results.

**Table 3.** Expression pattern of tumor antigens in various tissues (selection). Northern-blot analysis was performed with RNA samples from tumor and normal human tissues matched by hybridization with GAPDH. Expression ratios were calculated after densitometric analysis of autoradiographs. The signal obtained with the normal counterpart of the diseased tissue was set to 1. n.t. = not tested;

antigen identity/homology	HOM-MEL-4C+	HOM-RCC-3.1.1.3+ CAH-like	HOM-GLIO-30.2.1 Tegt	HOM-HD-21+ Lectin-like
kidney	-	1	1.5	-
brain	-	n.t.	1	n.t.
tonsil	-	-	1	1
stomach	-	-	1.5	-
colon mucosa	-	0.2	1.5	-
breast	-	-	1.0	-
renal cancer	>5 in 4/19 cases ≤1 in 15/19 cases	n.t.	-	-
Hodgkin's tissue	n.t.	-	n.t.	>10
astrocytoma	n.t.	n.t.	>5 in 8/12 1 in 4/12	-
melanoma	++	-	n.t.	-

As will be seen, the new melanoma associated antigen is strongly expressed in melanoma, but not other tissues. Carbonic-anhydrase-like antigen was strongly expressed in about 20% of renal cell carcinomas, and only weakly in normal renal tissue. Tegt was overexpressed on 8/12 astrocytoma tissues compared to normal brain tissue. The mRNA for the lectin like molecule associated with Hodgkin's disease was increased about ten fold in diseased tonsils as compared to normal tonsils, suggesting that overexpression may be a frequent characteristic of proteins which elicit autologous B cell responses.

#### Example 10

Further studies were carried out on the HOM-MEL-40 sequence. Using standard genetic analysis techniques, the 5' region of the mRNA for HOM-MEL 40 was shown to have a tyrosine kinase binding domain. This suggests that HOM-MEL-40 may function as a receptor. The 3' portion of the RNA is identical with an RNA molecule for "SSX," a molecule known to be involved in the SYT-SSX translocation in synovial tumors.

#### Example 11

Additional experiments were also carried out to study HOM-MEL 40. Standard Northern blotting showed that, with the exception of testis, HOM-MEL 40 was not expressed in normal tissues. In contrast, it was expressed in 50% of melanomas, 20% of prostate cancers, 20% of gastric cancers, 26% of colorectal cancers, 12% of the lung cancers and 20% of breast and hepatocellular cancer. It was also found in 1/10 gastric, and 1/5 thyroid carcinomas.

Additional Western blotting work was carried out, showing that antibodies against HOM-MEL 40 were present in 10 of 89 melanoma patients tested, but only 3 out of 49 healthy male subjects.

In yet further studies, it was observed that HLA-A2 positive tumor cells presented a nonamer derived from HOM-MEL. This suggests that HOM-MEL 40 specific vaccines, useful in inducing CTLs, are possible.

Example 12

The phage assay described in the prior examples is not appropriate for screening large numbers of serum samples. In order to do so, a modification of the standard Western Blot was developed. This variation is based upon His-tagged, recombinant HOM-MEL 40, as is herein described.

HOM-MEL-40 was amplified, over 20 cycles, using pfu polymerase, on plasmid cDNA prepared from melanoma tissue. The oligonucleotide primers used were:

10     5'-GCCAAATACTTCTCTAAGGAAGAGTGG-3'     (SEQ ID NO: 4); (sense)  
5'-TTCACTGTTGTGAACACTTGCTTTCAC-3     (SEQ ID NO: 5);  
         (antisense)

15     Polymerase chain reaction (PCR) was carried out at 95°C/1 minute; 60°C/1 minute; and 72°C/1 minute, followed by a final extension at 72°C for 10 minutes. The amplification product was gel purified, using art recognized techniques, and then ligated in frame to SmaI digested, dephosphorylated and gel purified pQE32 vector. This results in production of a fusion protein having a "tail" of 6 histidine molecules at the N-terminus.

20     The construct was then transformed into E. coli SG13009 (pREP4) strain, followed by selection on plates containing kanamycin and ampicillin. Individual colonies were picked, and expressed on a small scale by inducing these with 2 mM isopropyl thiogalactoside (IPTG). This permits checking for protein expression. Small scale purification over Ni-NTA columns was then performed, for each clone.

25     One clone was identified as expressing a protein of expected length. This clone was isolated and sequenced using well known techniques. It was verified as HOM-MEL-40. Following the identification, large scale induction of recombinant protein was carried out. Specifically, cells were induced with 2 mM IPTG, and harvested five hours later. Cells were lysed, by combining with a buffer of 8 M urea, 100 mM Na<sub>2</sub>PO<sub>4</sub>, 10 mM Tris-HCl (pH8), 0.01% Triton X, overnight. Any

30

35

cellular debris was spun down, and supernatant was loaded onto pre-equilibrated Ni-NTA resin. Washes were performed with two volumes of the aforementioned buffer, at pH8, and then at least 10 volumes of the buffer, at pH6.3. Protein was then eluted, using the buffer described herein, plus 250 mM imidazole. Yields ranged from 15 to 40 mg of His-tagged protein per liter of bacterial culture.

Western Blotting was then carried out, using the His-labeled protein. In these assays, 5 ug of recombinant His-tagged proteins, which served as internal negative controls, were mixed with 2xSDS sample buffer (0.1 M Tris-HCl, pH 6.8, 0.2M dithiothreitol, 4% SDS, 0.2% bromophenol blue, 20% glycerol), and were then electrophoresed in 12% SDS-PAGE, followed by blotting to nylon membranes using semi-dry transfer.

After blocking of any unspecified binding with 5% low fat milk in PBS (1 hour), membranes were incubated with 1:100 diluted sera from tumor patients or healthy controls. Blots were then incubated for one hour with alkaline phosphatase conjugated mouse anti-human IgG. The membrane was then incubated, consecutively, with rabbit anti-mouse IgG (30 minutes), anti-alkaline phosphatase, and then 0.25 mg/ml of alkaline phosphatase. After each incubation step, the membranes were washed extensively with TBS and 0.1% Tween. Visualization was performed by staining with 5-bromo-4-chloro-3-indolyl-phosphate (BCIP), and nitroblue tetrazolium. The sera were analyzed in random order (healthy/melanoma positive), with the observer blind to the status of the sample. All analyses were carried out in duplicate.

The blotting showed a product with a molecular weight of about 24 kD by SDS-PAGE, which is consistent with a calculated molecular weight of 21.6 kD, based upon predicted amino acid sequence.

The immunoblotting described supra, was carried out on 89 melanoma samples, six ovarian cancer samples, and ten renal carcinoma samples. Of these, 11 melanoma samples, one ovarian cancer sample, and three renal cell carcinoma samples were



positive. Sera from subjects with colorectal, lung, breast, gastric, or pancreatic cancer were negative. A total of 41 healthy controls were also analyzed, of which three were positive. Any sera which were reactive in the Western blot, as well as twenty negative serum samples were then reassessed, using the phage assay, described supra. The reactivity was confirmed for 10 of the 11 melanoma patients and the positive ovarian cancer patient. The renal cell cancer patient and the healthy controls were negative, and were all samples negative in the Western blot.

There were 16 melanoma patients who provided both serum and tumor specimens. It was found that HOM-MEL-40 expression by the tumor could be compared with antibody reactivity in the sample. As will be seen in the table which follows, eight of the sixteen patients had HOM-MEL-40 positive tumors, but only three had antibodies against the antigen in their serum. No antibodies were detected in the serum of patients with HOM-MEL-40 negative tumors.

#### Example 13

Rammensee, et al., Immunogenetics 41: 178-228 (1995), incorporated by reference, disclose many peptides which bind to HLA-2.1, and some which also provoke CTL proliferation. Some of these are of formula:

XaaLeu(Xaa)<sub>6</sub>(Ile,Leu,Val)

(SEQ ID NO: 6). The deduced amino acid sequence for HOM-MEL-40 was screened for sequences which might act as HLA-A2.1 binders/CTL stimulators, and the following were found:

Arg Leu Gln Gly Ile Ser Pro Lys Ile (SEQ ID NO: 7);

Arg Leu Arg Glu Arg Lys Gln Leu Val (SEQ ID NO: 8);

Lys Ile Gln Lys Ala Phe Asp Asp Ile (SEQ ID NO: 9)

These peptides were synthesized, using well known techniques and Fmoc protected amino acids. The peptides were then purified, using Sephadex G25, followed by reverse phase HPLC on a C-18 column. T2 cells, which are deficient in transporters associated with antigen presentation (DeMars, et al., Proc. Natl. Acad. Sci. USA 82: 8183-8187 (1985); Slater, et al., Immunogenetics 21: 235-241 (1990)), were used in

peptide binding assays, as follows. A sample of  $5 \times 10^5$  T2 cells were incubated for 4 hours at 37°C, in the presence or absence of 100  $\mu$ M of the HOM-MEL-40 peptides. Positive controls were an EBV LMP2 derived peptide:

5 Cys Leu Gly Gly Leu Leu Thr Met Val (SEQ ID NO: 10)  
and an HIV reverse transcriptase derived peptide:

Ile Leu Lys Glu Pro Val Gly Val (SEQ ID NO: 11)

Upregulation of HLA-A2.1 on T2 cells was measured by labelling  
with an anti-HLA-A2.1 specific monoclonal antibody (i.e.,  
10 BB7.2), followed by incubation with an FITC conjugated goat  
anti-mouse antibody. The samples were analyzed by flow  
cytometry, with upregulation of HLA-A2.1 being given by the  
ratio:

15 
$$\frac{\text{mean fluorescence intensity in sample with peptide}}{\text{mean fluorescence intensity in sample without peptide}}$$

Each of the three peptides were seen to bind to HLA-A2.1, with  
20 SEQ ID NO: 8 showing strongest HLA-A2.1 upregulation by far;  
as determined by FACS analysis.

As the foregoing shows, the invention relates to a method  
for determining or isolating an immunoreactive substance.  
"Immunoreactive substance" as used herein refers to any  
25 material which provokes some form of immune response in the  
subject which produces it. This response may be based upon  
either a B cell or a T cell response. Such immunoreactive  
substances include proteins, peptides, glycoproteins,  
lipoproteins, peptide containing complexes (e.g., MHC/peptide  
30 complexes), antibodies, and so forth. To determine such  
substances, a cDNA library is prepared from cells of a  
subject, using well known, standard methods. The cDNA is then  
inserted into an appropriate vector, such as a eukaryotic cell  
specific virus or a phage (i.e., a bacterial virus), to form  
35 a transfecting/transforming library, which is then  
incorporated into a host cell. The host cells are treated so

that they express the library component (cloned cDNA) they receive. The host cells are then lysed, so that the expressed material is available for further treatment.

5 The lysed material is then contacted with a "stripped" sample believed to contain an immunogenic binding partner for the immunoreactive substance. "Immunogenic binding partner" as used herein refers to any immune system associated material which binds to the target, i.e., the immunoreactive substance. Such binding partners include, but are not limited to, 10 antibodies, T cells, cytokines, ligands, receptors, and so forth, as well as truncated portions of these molecules, complementary nucleic acid molecules, and so forth. Note that for some of these components, such as T cells, further steps including those recited herein are required.

15 The stripped sample, as indicated supra, has been treated by contact with both (i) non-transfected or transformed host cells, and (ii) host cells transfected or transformed with vectors which do not contain the pertinent cDNA.

20 The stripped sample is useful for identifying binding partners for the expressed material because many of the immune components which would otherwise interfere with the specific immunological reaction desired have been removed via the absorption steps described herein.

25 The identification of the expressed material may be followed by isolation of the cDNA encoding it. One can punch holes through a membrane such as a nitrocellulose membrane, placed on top of Petri dishes containing colonies of host cells, then use the immune reaction to give position on the solid phase. Each colony is based upon limited cDNA 30 transfection, thereby facilitating isolation and identification of relevant cDNA.

35 The invention also relates to the isolated nucleic acid molecules of SEQ ID NO: 1, 2 or 3, which encode for molecules which are associated with particular conditions. In addition to their role as coding materials, these molecules can also be used as probes to identify cells expressing the relevant antigens, as it has been shown that these cDNA molecules (SEQ

ID NO: 1, 2 and 3) are based upon mRNA which translated to the antigen.

Also a part of the invention are isolated nucleic acid molecules, the complementary sequences of which hybridize to one of SEQ ID NO: 1, 2 or 3, and which encode a protein equivalent to those encoded by SEQ ID NO: 1, 2 or 3. "Stringent conditions" as used herein, refers to conditions at least as stringent as hybridization at 50  $\mu$ l/cm<sup>2</sup> of 3.5xSSC, 1xDenhardt's solution, 25 mM sodium phosphate buffer (pH 7.0), using a <sup>32</sup>P-labelled probe, for 18 hours at 65°C, followed by four washes (one hour, each wash, at 65°C, 2xSSC, 0.1% SDS), and a final wash for 30 minutes at 1.0xSSC 0.2% SDS. The final wash can be changed to 0.5xSSC to 0.2xSSC, or even 0.1xSSC, and SDS can be lowered to 0.1% to increase stringency, if desired.

The invention also includes those peptides associated with tumor antigens, such as those of SEQ ID NOS: 7, 8 and 9, which bind to HLA-A2.1 molecules, thereby provoking lysis by cytolytic T cells. Also a part of the invention are peptides of formula

Xaa Leu Xaa, (SEQ ID NO: 12)

wherein the sixth amino acid residue is Ser, Lys or Phe, and the ninth amino acid residue is Val or Ile. These molecules can also serve, very simply, as markers for HLA-A2.1 cells, as it is well known that peptide/MCH complex formation is quite specific.

Other features of the invention will be clear to the skilled artisan and need not be reiterated here.

The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, it being recognized that various modifications are possible within the scope of the invention.

## (1) GENERAL INFORMATION:

- (i) APPLICANTS: Pfreundschuh, Michael; Rammensee, Hans-Georg
- (ii) TITLE OF INVENTION: Method For Identifying Or Isolating A Molecule And Molecules Identified Thereby
- (iii) NUMBER OF SEQUENCES: 13
- (iv) CORRESPONDENCE ADDRESS:
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  - (C) CITY: New York City
  - (D) STATE: New York
  - (F) ZIP: 10022
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Diskette, 3.5 inch, 360 kb storage
  - (B) COMPUTER: IBM
  - (C) OPERATING SYSTEM: PC-DOS
  - (D) SOFTWARE: Wordperfect
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER: 08/644,116
  - (B) FILING DATE: 10-MAY-1996
  - (C) CLASSIFICATION: 435
- (vi) PRIOR APPLICATION DATA:
  - (A) APPLICATION NUMBER: 08/580,980
  - (B) FILING DATE: 03-JANUARY-1996
- (vii) PRIOR APPLICATION DATA:
  - (A) APPLICATION NUMBER: 08/479,328
  - (B) FILING DATE: 07-JUNE-1995
- (viii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: Hanson, Norman D.
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  - (C) REFERENCE/DOCKET NUMBER: LUD 5410.2
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- (2) INFORMATION FOR SEQUENCE ID NO: 1:  
 (i) SEQUENCE CHARACTERISTICS:  
     (A) LENGTH: 2679 base pairs  
     (B) TYPE: nucleic acid  
     (C) STRANDEDNESS: double-stranded  
     (D) TOPOLOGY: linear  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

CGCGAAGATG	CCCCGGCGCA	GCCTGCACGC	GGCGGCCGTG	CTCCTGCTGG	50
TGATCTTAAA	GGAACAGCCT	TCCAGCCCGG	CCCCAGTGAA	CGGTTCCAAG	100
TGGACTTATT	TTGGTCCTGA	TGGGGAGAAT	AGCTGGTCCA	AGAAGTACCC	150
GTCGTGTGGG	GGCCTGCTGC	AGTCCCCCAT	AGACCTGCAC	AGTGACATCC	200
TCCAGTATGA	CGCCAGCCTC	ACGCCCTCG	AGTTCCAAGG	CTACAATCTG	250
TCTGCCAACA	AGCAGTTTCT	CCTGACCAAC	AATGGCCATT	CAGTGAAGCT	300
GAACCTGCCC	TCGGACATGC	ACATCCAAGG	CCTCCAGTCT	CGCTACAGTG	350
CCACGCAGCT	GCACCTGCAC	TGGGGGAACC	CGAATGACCC	GCACGGCTCT	400
GAGCATAACG	TCAGCGGACA	GCACCTTCTC	GCCGAGCTGC	ACATTGTCCA	450
TTATAACTCA	GACCTTTATC	CTGACGACAG	NACTGCCAGC	AACAAGTCAG	500
AAGACCTCGC	TGTCCTGGGT	GCTCTCATTG	AGATGGGCTC	CTTCAATCCG	550
TCCTATGACA	AGATCTTCAG	TCACCTTCAA	CATGTAAAGT	ACAAAGGCCA	600
GGAAGCATTG	GTCCCGGGAT	TCAACATTGA	AGAGCTGCTT	CCGGAGAGGA	650
CCGCTGAATA	TTACCGCTAC	CGGGGGTCCC	TGATCACACC	CCCTTGCAAC	700
CCCACTGTGC	TCTGGACAGT	TTTCCGAAAC	CCCCTGCAAA	TTTCCAGGA	750
GCAGCTGCTG	GCTTTGGAGA	CAGCCCTGTA	CTGCACACAC	ATGGACGACC	800
CTTCCCCCAG	AGAAATGATC	AACAACCTCC	GGCAGGTCCA	GAAGTTCGAT	850
GAGAGGCTGG	TATACACCTC	CTTCTCCCAA	GTGCAAGTGT	GTACTGCGGC	900
AGGACTGAGT	TGGGGCATCA	TCCTCTCACT	GGCCCTGGCT	GGCATTCTTG	950
GCATCTGTAT	TGTGGTGGTG	GTGTCCATTT	GGCTTTTCAG	AAGGAAGAGT	1000
ATCAAAAAAG	GTGATAACAA	GGGAGTCATT	TACAAGCCAG	CCACCAAGAT	1050
GGAGACTGAG	GCCCACGCTT	GAGGTCCCCG	GAGCTCCCCG	GCACATCCAG	1100
GAAGGACCTT	GCTTTGGACC	CTACACACTT	CGGCTCTCTG	GACACTTGCG	1150
ACACCTCAAG	GTGTTCTCTG	TAGCTCAATC	TGCAAAACATG	CCAGGCCTCA	1200
GGGATCTCT	GCTGGGTGCC	TCCTTGCTTT	GGGACCATGG	NCACCCAGAG	1250
GCCATCCGAT	CGATGGATGG	GATGCACTCT	CAGACCAAGC	AGCAGGAATT	1300
CAAGGCTGCT	TGCTGTAATT	GTGTGAGATT	GTGAAGTGGT	CTGAATTCTG	1350
GAATCACAAA	CCAACCATGC	TGGTGGGCCA	TTAATGGTTG	GAAAACACTT	1400
CCATCCGGGG	CTTTGCCAGA	GCCTGCTTTC	AAGTGTCTCT	GAAATTCTGC	1450
TGCTTCTCCA	AGCTTTCAGA	CAAGAATGTG	CACTCTCTGC	TTAGGTTTTG	1500
CTTGGGAAAC	TCAACTTCTT	TCCTCTGGAG	ACGGGACATC	TCCCTCTGAT	1550
TTCCTTCTGC	TATGCAAAAC	CTTTAATCTG	CACCTTACAN	ACTCGGGGAC	1600
AAATGGGGAC	AGGAAGGATC	AAGTTGTAGA	GNAGAAAAAG	AAAACAAGAG	1650
ATATACATTG	TGATATATAT	TAGGGACACT	TTCACAGTCC	TGTCCTCTGG	1700
ATCACAGACA	CTGCACAGAC	CTTAGGGAAA	TGGCAGGTTT	AAAGTTCCAC	1750
TTCTTGGTGG	GGATGAGAAG	GGAGAGAGAG	CTAGAGGGAC	AAAGAGAATG	1800
AGAAGACATG	GATGATCTGG	GAGAGTCTCA	CTTCGGAATC	AGAATTGGAA	1850
TCACATTCTG	TTTATCAAGC	CATAATGTAA	GGACAGAATA	ATACAATAAT	1900
AAGTCCAAAT	CCAACCTCCT	GTCAGTGGAA	CAGTTATGTT	TTATACTCTA	1950
CAGATTTTAC	AAATANATGA	GGCTNGTTCC	TTGAAAANTG	TGTTGNNTTG	2000
CTGTNGTCCN	NTGGAGGAGA	CATGAGTTCC	GAGATGACCA	ACTCNGNCNT	2050
TGNATNCTNG	GAGGNAATAN	GGCAGAACCA	AAATGACTGT	AGAACTTATT	2100
CTCTGTAGGC	CAAATTTTCA	TTCAGCCACT	TCTGCAGGAT	CCTACTGCCA	2150
ACCTGGAATG	GAGACTTTTA	TCTACTTCTC	TCTCTCTGAA	GATGTCAAAT	2200
CGTGTTTGTG	ATCAAAATATA	TTTCAAGCTA	TAAAAGCAGG	AGGTTATCTG	2250
TGCAGGGGGC	TGGCATCATG	TATTTAGGGG	CAAGTAATAA	TGGAATGCTA	2300
CTAAGATACT	CCATATTCTT	CCCCGAATCA	CACAGACAGT	TTCTGACAGG	2350

CGCAACTCCT	CCATTTTCCT	CCCGCAGGTG	AGAACCCTGT	GGAGATGAGT	2400
CAGTGCCATG	ACTGAGAAGG	AACCGACCCC	TAGTTGAGAG	CACCTTGCCAG	2450
TTCCCCGAGA	ACTTTCTGAT	TGCACAGTCT	CATTTTGACA	GCATGAAATG	2500
TCCTCTTGAA	GCATAGCTTT	TAAATATCT	TTTTCCTTCT	ACTCCTCCCT	2550
CTGACTCTAG	GAATTCTCTC	TTCTGGAATC	GCTTGAACCC	AGGAGGCGGA	2600
GGTTGCAGTA	AGCCAAGGTC	ATGCCACTGC	ACTCTAGCCT	GGGTGACAGA	2650
GCGAGACTCC	ATCTCAAAAA	AAAAAAAAAA			2679

## (2) INFORMATION FOR SEQUENCE ID NO: 2:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 931 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double-stranded

(D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

ACTTTCTCTC	TCTTTCGATT	CTTCCATACT	CAGAGTACGC	ACGGTCTGAT	50
TTTCTCTTTG	GATTCTTCCA	AAATCAGAGT	CAGACTGCTC	CCGGTGCCAT	100
GAACGGAGAC	GACGCCTTTG	CAAGGAGACC	CACGGTTGGT	GCTCAAATAC	150
CAGAGAAGAT	CCAAAAGGCC	TTGATGATA	TTGCCAAATA	CTTCTCTAAG	200
GAAGAGTGGG	AAAAGATGAA	AGCCTCGGAG	AAAATCTTCT	ATGTGTATAT	250
GAAGAGAAAG	TATGAGGCTA	TGACTAAACT	AGGTTTCAAG	GCCACCCTCC	300
CACCTTTCAT	GTGTAATAAA	CGGGCCGAAG	ACTTCCAGGG	GAATGATTTG	350
GATAATGACC	CTAACCGTGG	GAATCAGGTT	GAACGTCCTC	AGATGACTTT	400
CGGCAGGCTC	CAGGGAATCT	CCCCGAAGAT	CATGCCCCAG	AAGCCAGCAG	450
AGGAAGGAAA	TGATTGCGAG	GAAGTGCCAG	AAGCATCTGG	CCCACAAAAT	500
GATGGGAAAG	AGCTGTGCCC	CCCGGGAAAA	CCAACCTACCT	CTGAGAAGAT	550
TCACGAGAGA	TCTGGACCCA	AAAGGGGGGA	ACATGCCTGG	ACCCACAGAC	600
TGCGTGAGAG	AAAACAGCTG	GTGATTTATG	AAGAGATCAG	CGACCCTGAG	650
GAAGATGACG	AGTAACTCCC	CTCAGGGATA	CGACACATGC	CCATGATGAG	700
AAGCAGAACG	TGGTGACCTT	TCACGAACAT	GGGCATGGCT	GCGGACCCCT	750
CGTCATCAGG	TGCATAGCAA	GTGAAAGCAA	GTGTTACAAA	CAGTGAAAAA	800
TTGAGCGTCA	TTTTTCTTAG	TGTGCCAAGA	GTTGATGTT	AGCGTTTACG	850
TTGTATTTTC	TTACACTGTG	TCATTCTGTT	AGATACTAAC	ATTTTCATTGA	900
TGACGAAGAC	ATACTTAATC	GATATTTGGT	T		931

## (2) INFORMATION FOR SEQUENCE ID NO: 3:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1692 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double-stranded

(D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GATCCCCCGG	GCTGCAGGAA	TTGCGCACGA	GCAAAGGACT	TCCTAGTGGG	50
TGTGAAAGGC	AGCGGTGGCC	ACAGAGGCGG	CGGAGAGATG	GCCTTCAGCG	100
GTTCCAGGCG	TCCCTACCTG	AGTCCAGCTG	TCCCCTTTTC	TGGGACTATT	150
CAAGGAGGTC	TCCAGGACGG	ACTTCAGATC	ACTGTCAATG	GGACCGTTCT	200
CAGTCCAGT	GGAACCAGGT	TTGCTGTGAA	CTTTCAGACT	GGCTTCAGTG	250
GAAATGACAT	TGCCTTCCAC	TTCAACCCTC	GGTTTGAAGA	TGGAGGGTAC	300
TTGGTGTCCA	ACACGAGGCA	GAACGGAAGC	TGGGGGCCCG	AGGAGAGGAA	350

GACACACATG	CCTTNCCAGA	AGGGGATGCC	CTTTGACCTC	TGCTTCCTGG	400
TGCAGAGCTC	AGATTTCAAG	GTGATGGTGA	ACGGGATCCT	CTTCGTGCAG	450
TACTTCACAT	CTCGTCATGC	CCTGTCCACC	GTTGTGGACA	CCATCTCCGT	500
CAATGGCTCT	GTGCAGCTGT	CCTACATCAG	CTTCCAGCCT	CCCGGCGTGT	550
GGCCTGCCAA	CCCGGCTCCC	ATTACCCAGA	CAGNNNTCAT	CCACACAGTN	600
GCAGAGCGCC	CNCTGGACAG	ATGTCTCTAC	TCCCGCCATC	CCACCTATGA	650
TGTACCCCCA	CCCCGCCTAT	CCGATGCCTT	TCATCACCAC	CATTCTGGGA	700
GGGCTGTACC	CATCCAAGTC	CATCCTCCTG	TCAGGCACTG	TNCTGCCCAG	750
TGCTCANGAG	GTTCCACATC	NAACCTGTGC	NCTGGGAACC	ACATCGCCTT	800
CCACCTGAAC	CCCCGTTTTG	ATGAGAAATG	TGTGGTCCGC	AACACCCAGA	850
TCGACAACTC	CTGGGGGTCT	CAGGAGCGAA	GTCTGCCCCG	AAAAATGCCC	900
TTCTGCCGTG	GCCAGAGCTT	CTCAGTGTGG	ATCTTGTGTG	AAGCTCACTG	950
CCTCAAGGTG	GCCGTGGATG	GTCAGCACCT	GTTTGAATAC	AACCATCGCC	1000
TGAGGAACCT	GCCCACCATC	AACAGACTGG	AAGTGGGGGG	CGACATCCAG	1050
CTGACCATGT	GCAGACATAG	GCGGCTTCCT	GGCCCTGGGG	CCGGGGGCTG	1100
GGGTGTGGGG	CAGTCTGGGT	CCTCTCATCA	TCCCCACTTC	CCAGGCCCAG	1150
CCTTTCCAAC	CCTGCCTGGG	ATCTGGGCTT	TAATGCAGAG	GCCATGTCCT	1200
TGTCTGGTCC	TGCTTCTGGC	TACAGCCACC	CTGGAACGGA	GAAGGCAGCT	1250
GACGGGGATT	GCCTCCTCAG	CCGACGACGC	ACCTGGGGCT	CCAGCTGCTG	1300
GAATCCTACC	ATCCCAGGAY	GCAGGCACAG	CCAGGGAGAG	GGGAGGNGTG	1350
GGCAGTGAAG	ATGAAGCCCC	ATGCTCAGT	CCCTCCCATC	CCCCACGCAG	1400
CTCCACCCCA	GTCCCAAGCC	ACCAGCTGTC	TGCTCCTGGT	GGGAGGTGGC	1450
CTCCTCAGCN	CCTCCTCTCT	GACCTTTAAC	CTNACTCTCA	CCTTGACCCG	1500
TGCACCAACC	CTTACCCCT	CCTGGAAAGC	AGGCCTGATG	GCTTCCCACT	1550
GGCTCCACC	ACCTGACCAG	AGTGTCTCT	TCAGAGGACT	GGCTCCTTTC	1600
CCAGTGTCT	TAAAATAAAG	AAATGAAAAT	NCTTGTGGC	AAAAAAAAAA	1650
AAAAAAAAAC	TCGAGGGGCG	NCCNGTACC	CAATTCGCC	TA	1692

## (2) INFORMATION FOR SEQUENCE ID NO: 4:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1240 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double-stranded

(D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

ATCTGCAGAA	TTCGGCTTCG	ATCTAGAACT	AGTGGATCCC	CCGGGCTGCA	50
GGAATTCGGC	ACGAGCGGTT	CCAAGTGGAC	TTATTTTGGT	CCTGATGGGG	100
AGAATAGCTG	GTCCAAGAAG	TACCCGTCGT	GTGGGGGCTT	GCTGCAGTCC	150
CCCATAGACC	TGCACAGTGA	CATCCTCCAG	TATGACGCCA	GCCTCACGCC	200
CCTCGAGTTC	CAAGGCTACA	ATCTGTCTGC	CAACAAGCAG	TTTCTCCTGA	250
CCAACAATGG	CCATTCACTG	AAGCTGAACC	TGCCCTCGGA	CATGCACATC	300
CAGGGCCTCC	AGTCTCGCTA	CAGTGCCACG	CAGCTGCACC	TGCACTGGGG	350
GAACCCGAAT	GACCCGCACG	GCTCTGAGCA	TACCGTCAGC	GGACAGCACT	400
TCTCCGCCGA	GCTGCACATT	GTCCATTATA	ACTCAGACCT	TTATCCTGAC	450
GACAGNACTG	CCAGCAACAA	GTGAGAAGAC	CTCGCTGTCC	TGGGTGCTCT	500
CATTGAGATG	GGCTCCTTCA	ATCCGTCTTA	TGACAAGATC	TTCAGTCACC	550
TTCAACATGT	AAAGTACAAA	GGCCAGGAAG	CATTCTGCCC	GGGATTCAAC	600
ATTGAAGAGC	TGCTTCCGGA	GAGGACCGCT	GAATATTACC	GCTACCGGGG	650
GTCCCTGATC	ACACCCCTTT	GCAACCCAC	TGTGCTCTGG	ACAGTTTTC	700
GAAACCCCGT	GCAAATTTCC	CAGGAGCAGC	TGCTGGCTTT	GGAGACAGCC	750
CTGTACTGCA	CACACATGGA	CGACCTTCC	CCCAGAGAAA	TGATCAACAA	800
CTNCCGGCAG	GTCCAGAAGT	TCGNTGAGAG	GCTGGTATAC	ACCTCCTTCT	850



31

CNCAAGTGCA	AGTCTGTACT	GCGGCAGGAC	TGAGTCTGGG	CATCATCCTC	900
TCACTGGCCC	TGGCTGGCAT	TCTTGGCATC	TGTATTGTGG	TGGTGGTGTC	1000
CATTTGGCTT	TTCAGAAGGA	AGAGTANCCC	CNAAAGGTGA	TAACAAGGGA	1050
GTCATTTACA	AGCCANCCAC	CAAGATGGAG	ACTGAGGCCC	ACGCTTGAGG	1100
TCCCCGGAGC	TCCCCGGGAC	ATCCAGGAAG	GACCTTGCTT	TTGGACCCTA	1150
CACACTTCGG	CTCTCTGGAC	ACTTGCGACA	CCTCAAGGTG	TTCTCTGTAG	1200
CTCAATCTGC	AAACATGCCA	GGCCTCAGGG	ATCCTCTGCT		1240

## (2) INFORMATION FOR SEQUENCE ID NO: 5:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GCCAAATACT TCTCTAAGGA AGAGTGG

27

## (2) INFORMATION FOR SEQUENCE ID NO: 6:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

TTCACTGTTG TGAACACTTG CTTTCAC

27

## (2) INFORMATION FOR SEQUENCE ID NO: 7:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

Xaa Leu Xaa Xaa Xaa Xaa Xaa Xaa

5

32

## (2) INFORMATION FOR SEQUENCE ID NO: 8:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

Arg Leu Gln Gly Ile Ser Pro Lys Ile

5

## (2) INFORMATION FOR SEQUENCE ID NO: 9:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

Arg Leu Arg Glu Arg Lys Gln Leu Val

5

## (2) INFORMATION FOR SEQUENCE ID NO: 10:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

Lys Ile Gln Lys Ala Phe Asp Asp Ile

5

## (2) INFORMATION FOR SEQUENCE ID NO: 11:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

Cys Leu Gly Gly Leu Leu Thr Met Val

5

## (2) INFORMATION FOR SEQUENCE ID NO: 12:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 8 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

Ile Leu Lys Glu Pro Val Gly Val

5

33

## (2) INFORMATION FOR SEQUENCE ID NO: 13:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ix) FEATURE: The sixth amino acid is Ser, Lys or Phe. The ninth

amino acid is Val or Ile

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

Xaa Leu Xaa Xaa Xaa Xaa Xaa Xaa Xaa

5

I claim:

1. Isolated nonapeptide which binds to an HLA-A2.1 molecule, and has fomrula

Xaa Leu Xaa7

wherein the sixth amino acid residue is Ser, Lys or Phe, and the ninth amino acid residue is Val or Ile.

2. The isolated nonapeptide of which 1, selected from the group consisting of:

SEQ ID NO: 7,

SEQ ID NO: 8, and

SEQ ID NO: 9.

3. The isolated nonapeptide of claim 1, which is SEQ ID NO: 7.

4. The isolated nonapeptide of claim 1, which is SEQ ID NO: 8.

5. The isolated nonapeptide of claim 1, which is SEQ ID NO: 9.

6. Isolated protein encoded by the nucleic acid molecule of SEQ ID NO: 1.

7 Method for screening for possible presence of a tumor associated protein, comprising contacting a sample with at least one of SEQ ID NO: 4 and SEQ ID NO: 5, and determining hybridization of SEQ ID NO: 4 or SEQ ID NO: 5 to a target, as a determination of said tumor associated antigen in said sample.

8. The method of claim 7, comprising contacting said sample with both SEQ ID NO: 4 and SEQ ID NO: 5.

9. The method of claim 8, comprising polymerase chain reaction.

10. Method for determining an immunoreactive substance produced by a subject, comprising:

- (a) producing a cDNA library of a cell taken from said subject,
- (b) inserting said cDNA library into a vector,
- (c) transfecting said vector into a host cell to produce a transfected host cell,
- (d) culturing said transfected host cell to express said immunoreactive substance,
- (e) lysing said transfected host cell to form a lysate,
- (f) contacting a sample containing an immunogenic binding partner for said immunoreactive substance with a sample of lysed, non-transfected host cells, to remove any immunogenic binding partner from said sample which is specific for said non-transfected host cell, to produce a stripped sample,
- (g) contacting said stripped sample to a sample of lysed host cells transfected with the same vector into which said cDNA has been inserted wherein said vector does not contain any cDNA, to remove any immunoreactive binding partners specific for said vector, thereby producing a twice stripped sample,
- (h) contacting said twice stripped sample to the lysate of (f), whereby any immunoreactive binding partners specific for said immunoreactive binding partner bind thereto, and
- (i) determining binding in (h) to determine said immunoreactive substance.

11. The method of claim 10, further comprising identifying the transfected host cell which expressed said immunoreactive substance, and isolating the cDNA contained therein.

12. The method of claim 10, further comprising removing any immunoreactive binding partner to isolate a specific immunoreactive binding partner for said substance.

13. The method of claim 10, wherein said host cell is E. coli.

14. The method of claim 10, wherein said vector is a viral vector.

15. The method of claim 14, wherein said viral vector is a eukaryotic virus based vector.

16. The method of claim 14, wherein said viral vector is a phage vector.

17. The method of claim 16, wherein said phage is lambda phase.

18. The method of claim 10, wherein said host cell is a prokaryote.

19. The method of claim 10, wherein said host cell is a eukaryote.

20. The method of claim 10, further comprising immobilizing said lysate to a solid phase prior to contact with said twice stripped sample.

21. The method of claim 10, wherein said sample is serum.

22. The method of claim 21, wherein said serum is autologous serum.

23. The method of claim 10, wherein said immunogenic binding partner is an antibody.

24. The method of claim 10, wherein said immunoreactive substance is an antigen associated with cancer.

25. The method of claim 10, wherein said immunoreactive substance is an autoimmune associated antigen.

26. The method of claim 10, wherein said immunoreactive substance is produced by a pathogen.

27. Isolated nucleic acid molecule which codes for a renal cell carcinoma-specific antigen, the complementary sequence of which hybridizes to SEQ ID NO: 1, under stringent conditions.

28. Isolated nucleic acid molecule which codes for a Hodgkin's Disease-specific antigen, the complementary sequence of which hybridizes to SEQ ID NO: 2 under stringent conditions.

29. Isolated nucleic acid molecule which codes for a melanoma specific antigen, the complementary sequence of which hybridizes to SEQ ID NO: 3 under stringent conditions.

30. Isolated nucleic acid molecule of claim 27, consisting of SEQ ID NO: 1.

31. Isolated nucleic acid molecule of claim 28, consisting of SEQ ID NO: 2.

32. Isolated nucleic acid molecule of claim 29, consisting of SEQ ID NO: 3.

33. Expression vector comprising the isolated nucleic acid molecule of claim 27, operably linked to a promoter.

34. Expression vector comprising the isolated nucleic acid molecule of claim 28, operably linked to a promoter.

35. Expression vector comprising the isolated nucleic acid molecule of claim 29, operably linked to a promoter.

36. Cell line transformed or transfected with the isolated nucleic acid molecule of claim 27.

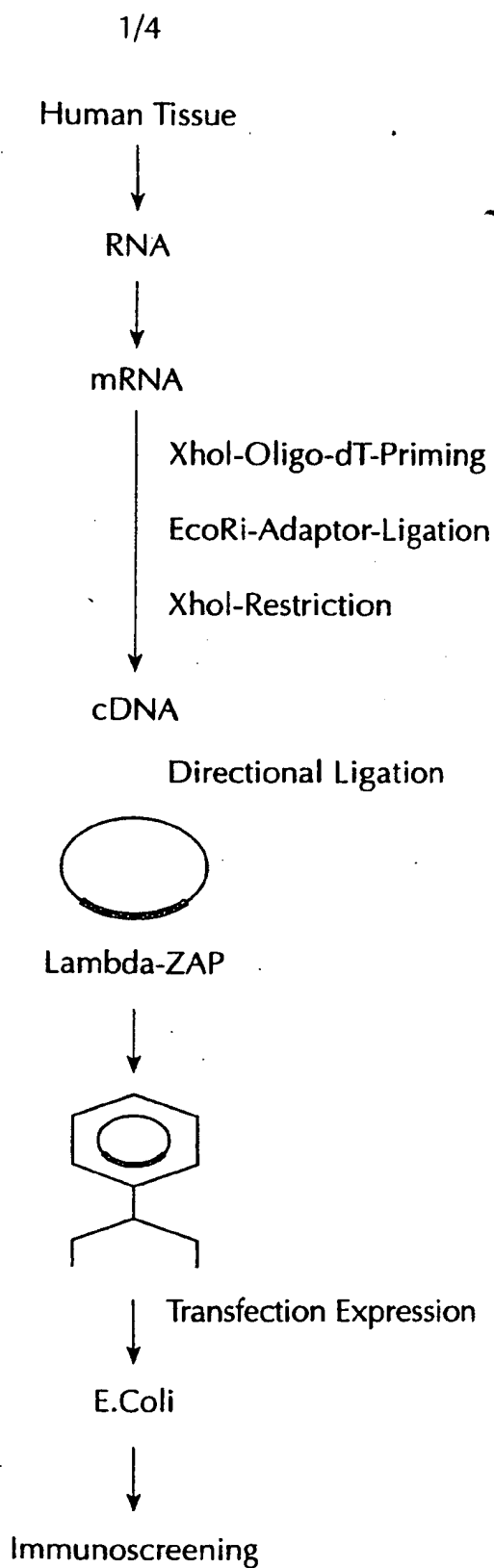
37. Cell line transformed or transfected with the isolated nucleic acid molecule of claim 28.

38. Cell line transformed or transfected with the isolated nucleic acid molecule of claim 29.

39. Cell line transformed or transfected with the expression vector of claim 33.

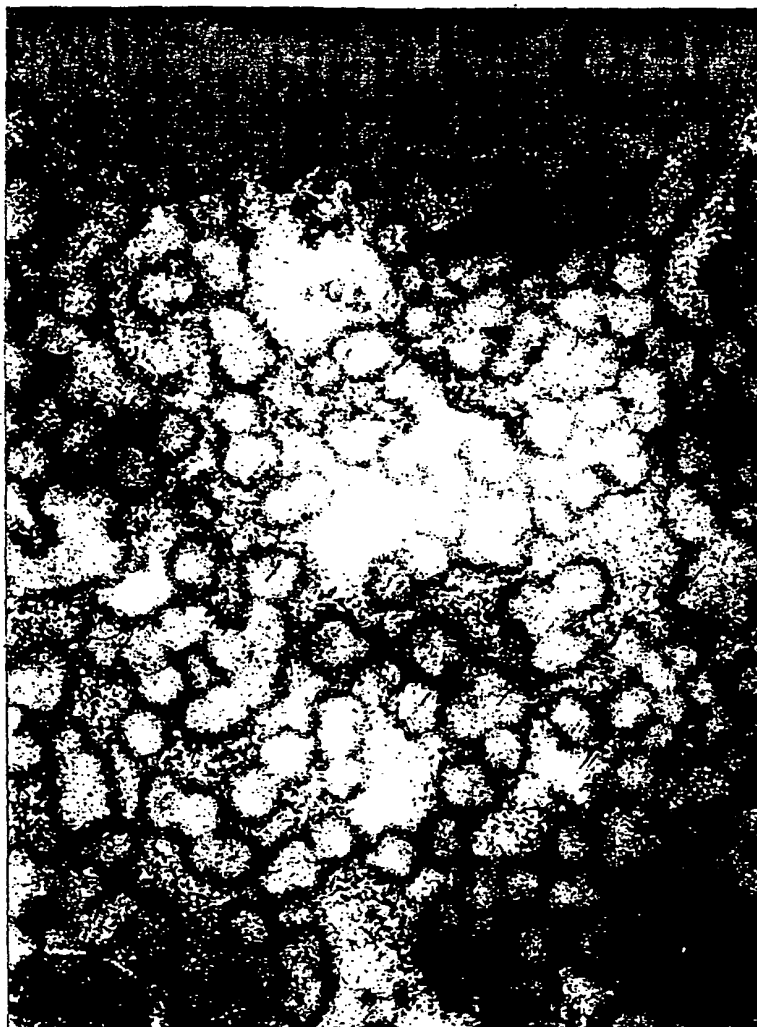
40. Cell line transformed or transfected with the expression vector of claim 34.

41. Cell line transformed or transfected with the expression vector of claim 35.

**FIG. 1**

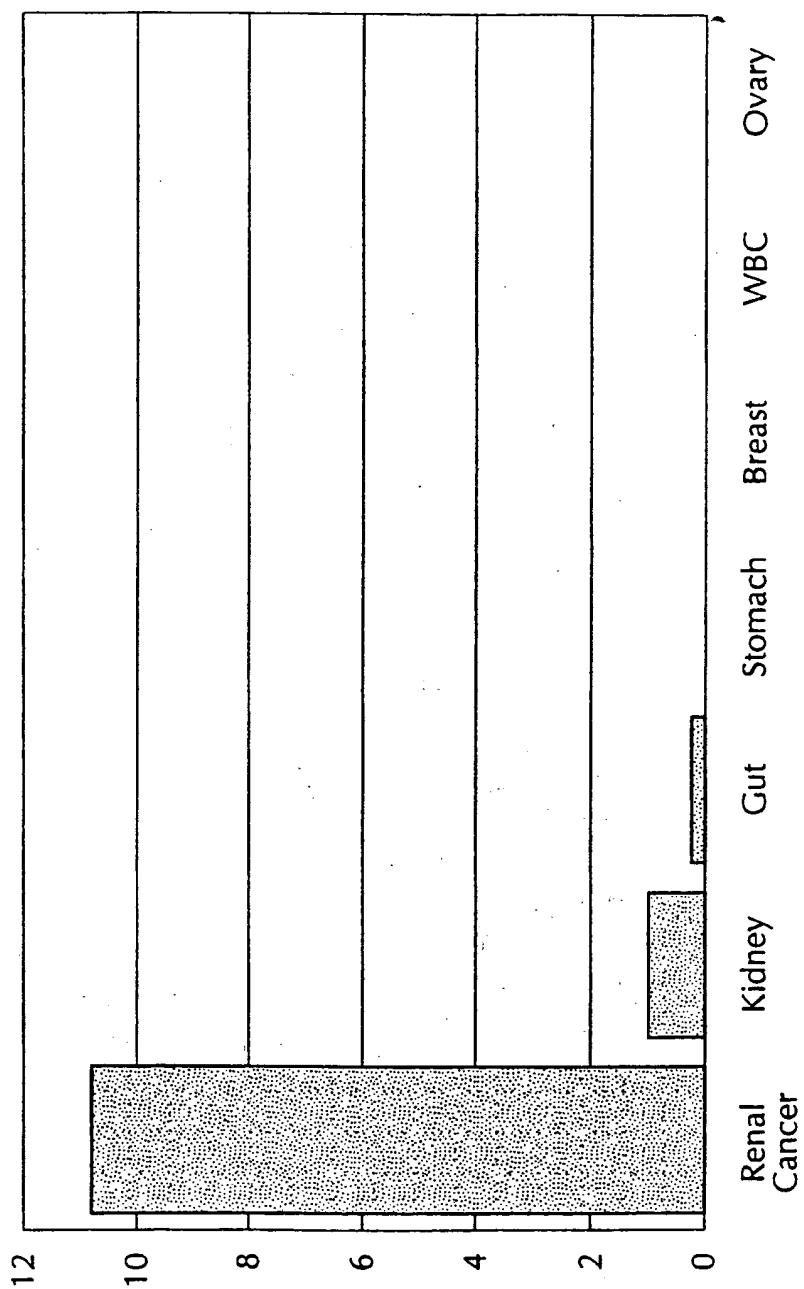


**FIG. 2**



3/4

FIG. 3



4/4

ORIGIN

1 ATCTGCAGAA TTCGGCTTCG ATCTAGAACT AGTGGATCCC CCGGGCTGCA GGAATTCCGGC  
 61 ACGAGCGGTT CCAAGTGGAC TTATTTTGGT CCTGATGGGG AGAATAGCTG GTCCAAGAAG  
 121 TACCCGTCGT GTGGGGCCT GCTGCAGTCC CCCATAGACC TGCACAGTGA CATCCTCCAG  
 181 TATGACGCCA GCCTCACGCC CCTCGAGTTC CAAGGCTACA ATCTGTCTGC CAACAAGCAG  
 241 TTTCTCCTGA CCAACAATGG CCATTCAGTG AAGCTGAACC TGCCCTCGGA CATGCACATC  
 301 CAGGGCCTCC AGTCTCGCTA CAGTGCCACG CAGCTGCACC TGCAC TGGG GAACCCGAAAT  
 361 GACCCGCACG GCTCTGAGCA TACCGTCAGC GGACAGCACT TCTCCGCCGA GCTGCACATT  
 421 GTCCATTATA ACTCAGACCT TTATCCTGAC GACAG?ACTG CCAGCAACAA GTCAGAAGAC  
 481 CTCGCTGTCC TGGGTGCTCT CATTGAGATG GGCTCCTTCA ATCCGTCCTA TGACAAGATC  
 541 TTCAGTCACC TTCAACATGT AAAGTACAAA GGCCAGGAAG CATTCTGTCCC GGGATTCAAC  
 601 ATTGAAGAGC TGCTTCCGGA GAGGACCGCT GAATATTACC GCTACCGGGG GTCCCTGATC  
 661 ACACCCCTT GCAACCCAC TGCTCTCTGG ACAGTTTTC GAAACCCCGT GCAAATTTC  
 721 CAGGAGCAGC TGCTGGCTTT GGAGACAGCC CTGTACTGCA CACACATGGA CGACCCCTTC  
 781 CCCAGAGAAA TGATCAACAA CT?CCGGCAG GTCCAGAAAGT TCG?TGAGAG GCTGGTATAC  
 841 ACCTCCTTCT C?CAAGTGCA AGTCTGTACT GCGGCAGGAC TGAGTCTGGG CATCATCCTC  
 901 TCACTGGCCC TGGCTGGCAT TCTTGGCATC TGTATTGTGG TGGTGGTGTG CATTTGGCTT  
 961 TTCAGAAGGA AGAGTA?CCC C?AAAGTGA TAACAAGGGA GTCATTTACA AGCCA?CCAC  
 1021 CAAGATGGAG ACTGAGGCC ACGCTTGAGG TCCCGGGGAC TCCCGGGCAC ATCCAGGAAG  
 1081 GACCTTGCTT TTGGACCCCTA CACACTTCGG CTCTCTGGAC ACTTGCGACA CCTCAAGGTG  
 1141 TTCTCTGTAG CTCAAATCTGC AAACATGCCA GGCTCAGGG ATCCTCTGCT

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FIG. 4

SUBSTITUTE SHEET (RULE 26)

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/09726

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 38/08; C07K 7/00; C12Q 1/68; G01N 33/53.

US CL : 530/328; 424/185.1, 277.1; 435/6, 7.1, 7.23, 7.24, 252.3

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/328; 424/185.1, 277.1; 435/6, 7.1, 7.23, 7.24, 252.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
EXAMINER'S CELLULAR IMMUNOLOGY, MHC AND TUMOR REJECTION ANTIGEN FILES.

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

A-GENESEQ23, PIR47, SWISSPROT-32, EMBLNEW-3, EST-STS.

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 5,405,940 (BOON ET AL.) 11 April 1995, see entire document.	1-41
Y	Biochem. Biophys. Res. Comm. Vol. 202, No. 1, issued 15 July 1994, M. Ding et al., "Cloning and analysis of MAGE-1-related genes, see entire document.	1-41

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

Special categories of cited documents:	
*A* document defining the general state of the art which is not considered to be of particular relevance	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*E* earlier document published on or after the international filing date	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*O* document referring to an oral disclosure, use, exhibition or other means	*G* document member of the same patent family
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

08 AUGUST 1996

Date of mailing of the international search report

26 SEP 1996

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